

Acta Medica Okayama

Volume 35, Issue 3

1981

Article 3

JUNE 1981

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Abstract

Generation of superoxide and hydrogen peroxide during interaction of nitrite with human hemoglobin was detected by chemiluminescence of luminol. Luminol chemiluminescence was inhibited by the addition of superoxide dismutase (SOD) and catalase. Methemoglobin formation induced by nitrite was also inhibited by the addition of SOD and catalase. The mechanism of methemoglobin formation by nitrite was discussed in regard to the oxidation of hemoglobin by superoxide and hydrogen peroxide as generated by the interaction of nitrite with hemoglobin.

KEYWORDS: nitrite, chemiluminescence, methemoglobin, superoxide dismutase.

*PMID: 6270983 [PubMed - indexed for MEDLINE]

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GENERATION OF SUPEROXIDE AND HYDROGEN PEROXIDE DURING INTERACTION OF NITRITE WITH HUMAN HEMOGLOBIN

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Received January 30, 1981

Abstract. Generation of superoxide and hydrogen peroxide during interaction of nitrite with human hemoglobin was detected by chemiluminescence of luminol. Luminol chemiluminescence was inhibited by the addition of superoxide dismutase (SOD) and catalase. Methemoglobin formation induced by nitrite was also inhibited by the addition of SOD and catalase. The mechanism of methemoglobin formation by nitrite was discussed in regard to the oxidation of hemoglobin by superoxide and hydrogen peroxide as generated by the interaction of nitrite with hemoglobin.

Key words : nitrite, chemiluminescence, methemoglobin, superoxide dismutase.

Sodium nitrite is the raw material of nitrochemicals and is well known as the causative agent of many industrial poisonings (1). Nitric oxide is the toxic substance of air pollutants and changes into nitrite and nitrate when dissolved in alkaline solution (2). Inhaled nitric oxide primarily reacts with hemoglobin and changes to nitrite and nitrate (3). It is well known that nitrite strongly forms methemoglobin *in vivo* and *in vitro*.

Kosaka *et al.* reported that oxyhemoglobin was oxidized by nitrite to form hemoglobin, nitrate and oxygen (4). However, the mechanism of methemoglobin formation induced by nitrite is still unclear.

Misra and Fridovich reported that superoxide radicals were generated during autooxidation of hemoglobin (5). Goldberg and Stern reported that superoxide radicals and hydrogen peroxide were related to the formation of methemoglobin induced by phenylhydrazine (6). Lynch *et al.* reported that superoxide dismutase inhibited the methemoglobin formation induced by superoxide which had been generated from photoreduced riboflavin (7). In this experiment, whether superoxide radicals and hydrogen peroxide were related to the formation of methemoglobin induced by nitrite was studied.

MATERIALS AND METHOD

Hemoglobin solution was prepared by the modified method of Aebi (8). Venous blood was obtained from a healthy young man and washed 3 times with cold saline. The erythrocyte layer was hemolysed by the addition of 9 volumes of cold redistilled water, then kept for an hour in the cold room and centrifuged at 10,000 r.p.m. for 40 min. Supernatant hemoglobin solution was dialyzed against distilled water for 24 h in the cold room.

DEAE-Sephadex(DE-52) made moist with 3 mM phosphate buffer (pH 6.9) was added to the hemolysate in order to adsorb catalase. Sephadex was discarded after centrifugation. Catalase free hemoglobin was thus obtained.

A Bioluminescence Reader BLR 101 B (Aloca Co) with recorder was used for the detection of luminol chemiluminescence. After initiation of the chemiluminescence reaction, a vial was placed in the sealed container of the BLR 101 B and the intensity of chemiluminescence followed with the recorder at sensitivity 3.

Catalase and SOD were injected into a vial through a needle as shown in Fig 3. Methemoglobin formation was measured by absorption at 630 m μ with an autorecording spectrophotometer (Hitachi Co.). All reactions were performed at 25°C. Milk xanthine oxidase (approximately 0.1 unit per mg protein), bovine liver catalase (37,000 Sigma units per mg protein) and bovine blood superoxide dismutase (approximately 3,000 units per mg protein) were purchased from Sigma Chemical Co.

RESULTS

Superoxide radicals and hydrogen peroxide were detected by the luminol chemiluminescence reaction (9). Superoxide radicals generated by a xanthine oxidase reaction oxidized luminol and generated chemiluminescence which was inhibited by the addition of superoxide dismutase as shown in Fig. 1.

Chemiluminescence which remained after the addition of SOD was assumed to be luminol chemiluminescence generated by oxidation with hydrogen peroxide which had been converted from superoxide by SOD.

Hydrogen peroxide oxidized luminol by a peroxidase-like activity of methemoglobin and generated chemiluminescence. This was completely inhibited by the addition of catalase as shown in Fig. 2.

A volume of 0.1 ml of 20 mM sodium nitrite was mixed with the reaction mixture containing 0.01 mM luminol and 0.15 mg/ml of hemoglobin in a total volume of 1.0 ml of 0.1 M phosphate buffer (pH 7.8) in the scintillation vial. This was placed in the sealed container of the Luminescence Reader. The chemiluminescence generated was followed with the recorder. chemiluminescence was inhibited by the addition of SOD and catalase as shown in Fig. 3.

It appeared that chemiluminescence was emitted by oxidation of luminol with superoxide and hydrogen peroxide which had been generated during the interaction of nitrite and hemoglobin. The maximum absorption peak appeared at 630 m μ by addition of sodium nitrite to the hemoglobin solution. This peak is specific for methemoglobin. The time course of methemoglobin formation curve was sigmoid. Methemoglobin formation was inhibited by the addition of catalase

and SOD as shown in Fig. 4. These data suggested that superoxide radicals and hydrogen peroxide were involved in the formation of methemoglobin induced by nitrite.

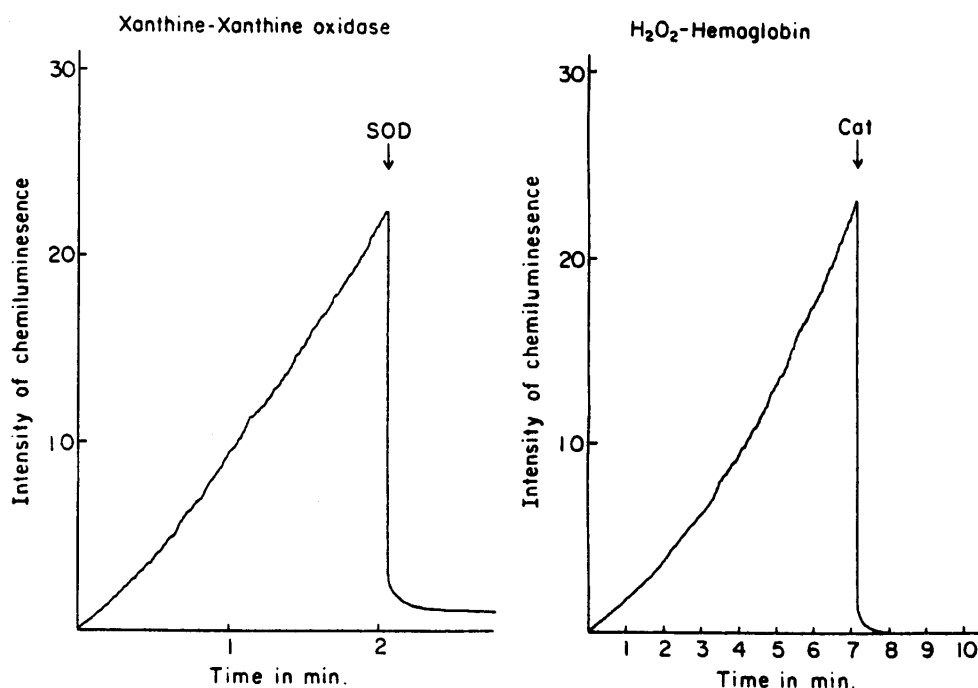


Fig. 1. (left) Chemiluminescence of luminol induced by superoxide which was generated by the xanthine xanthine oxidase reaction.

0.3 mM xanthine and xanthine oxidase (50 $\mu\text{g}/\text{ml}$) were mixed with 0.01 mM luminol in 0.1 M phosphate buffer (pH 7.8). Chemiluminescence was inhibited by the addition of SOD (10 $\mu\text{g}/\text{ml}$).

Fig. 2. (right) Chemiluminescence of luminol induced by hydrogen peroxide and hemoglobin.

0.01 mM hydrogen peroxide was mixed with 0.01 mM luminol and 0.15 mg/ml of hemoglobin solution in 0.1 M phosphate buffer (pH 7.8). Chemiluminescence was inhibited by the addition of catalase (10 $\mu\text{g}/\text{ml}$).

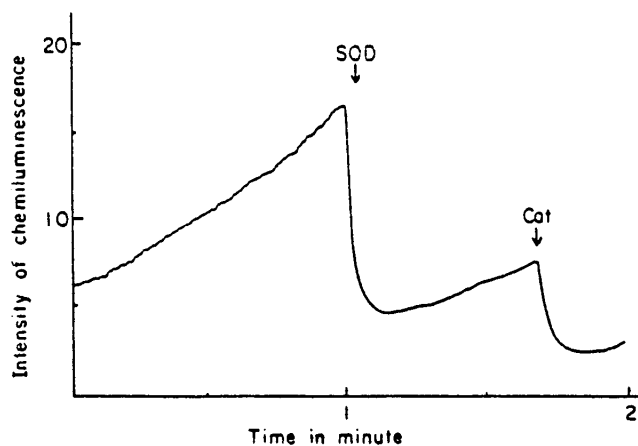


Fig. 3. Chemiluminescence of luminol induced by sodium nitrite and hemoglobin.

0.1 ml of 20 mM NaNO_2 was mixed with luminol and hemoglobin solution. Chemiluminescence was inhibited by the addition of SOD (10 $\mu\text{g/ml}$) and catalase (10 $\mu\text{g/ml}$).

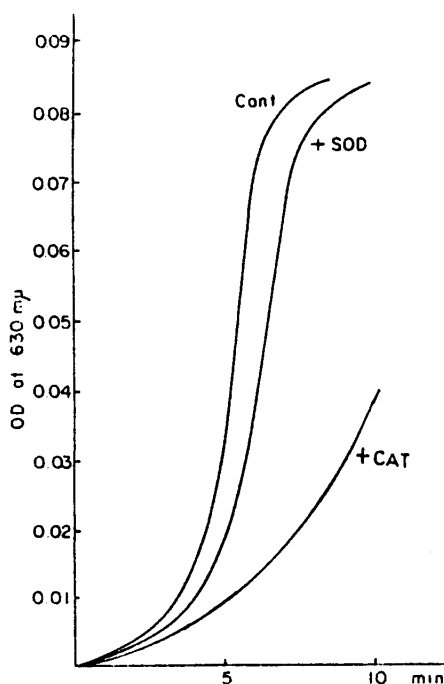


Fig. 4. Time course of methemoglobin formation induced by nitrite.

0.1 ml of 10 mM NaNO_2 was added to hemoglobin solution. The time course of methemoglobin formation was followed by absorption at an optical density of 630 $\text{m}\mu$ with an autorecording spectrophotometer. Methemoglobin formation was inhibited by the addition of SOD (30 $\mu\text{g/ml}$) and catalase (30 $\mu\text{g/ml}$).

DISCUSSION

Lynch *et al.* proposed that methemoglobin was formed when superoxide oxidized methemoglobin bound oxygen by one electron leading to dissociation of methemoglobin and hydrogen peroxide (10). It seemed possible that nitrite formed superoxide and hydrogen peroxide by a free radical chain reaction with hemoglobin much like that suggested by Misra and Fridovich for phenylhydrazine.(11).

In this experiment, luminol chemiluminescence generated by the interaction of nitrite with hemoglobin was inhibited by the addition of SOD and catalase. Consequently, it was suggested that superoxide and hydrogen peroxide were generated during the interaction of nitrite with hemoglobin.

Methemoglobin formation induced by nitrite was also inhibited by the addition of SOD and catalase. It appeared that superoxide and hydrogen peroxide oxidized hemoglobin to methemoglobin.

Catalase inhibited methemoglobin formation much more than SOD as shown in Fig. 3. This was mainly due to differences in the activity of SOD and catalase, the enzymes which were used in this experiment. Further experiments will be performed on the inhibition of methemoglobin formation by the coexistence of SOD and catalase.

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